Mammalian *Trithorax* and *Polycomb*-group homologues are antagonistic regulators of homeotic development

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Control of cell identity during development is specified in large part by the unique expression patterns of multiple homeobox-containing (*Hox*) genes in specific segments of an embryo. Trithorax and Polycomb-group (Trx-G and Pc-G) proteins in *Drosophila* maintain *Hox* expression or repression, respectively. Mixed lineage leukemia (MLL) is frequently involved in chromosomal translocations associated with acute leukemia and is the one established mammalian homologue of *Trx*. *Bmi-1* was first identified as a collaborator in *c-myc*-induced murine lymphomagenesis and is homologous to the Drosophila Pc-G member *Posterior sex combs*. Here, we note the axial-skeletal transformations and altered *Hox* expression patterns of *Mll*-deficient and *Bmi-1*-deficient mice were normalized when both *Mll* and *Bmi-1* were deleted, demonstrating their antagonistic role in determining segmental identity. Embryonic fibroblasts from *Mll*-deficient compared with *Bmi-1*-deficient mice demonstrate reciprocal regulation of *Hox* genes as well as an integrated *Hoxc8*-lacZ reporter construct. Reexpression of *MLL* was able to overcome repression, rescuing expression of *Hoxc8*-lacZ in *Mll*-deficient cells. Consistent with this, MLL and BMI-I display discrete subnuclear colocalization. Although *Drosophila* Pc-G and Trx-G members have been shown to maintain a previously established transcriptional pattern, we demonstrate that MLL can also dynamically regulate a target *Hox* gene.

Acute leukemias with cytogenetic abnormalities at chromosome segment 11q23 have a poor prognosis and bear a translocation of the mixed lineage leukemia (MLL) gene (1). *MLL*, a large protein (3,972 aa), is homologous to *Drosophila trithorax* (*trx*) in several regions. The homology between *MLL* and *trx* provided important clues about *MLL*'s normal function. The highest homology resides within a carboxy-terminal domain termed the SET domain because of its presence in the *Drosophila* proteins *Su(var)3–9*, *Enhancer of zeste*, and *trx* (2). SET domains are found in an evolutionarily conserved family of proteins that maintain specific patterns of gene expression after the initiation of transcription during development. Two major families of SET-containing proteins, the *Trithorax* (*trx*-G) and *Polycomb* (*Pc-G*) groups, are chromatin-associated proteins that act antagonistically to alter chromatin structure to either promote or repress transcription, respectively (3–5). Some of the best understood downstream targets of the *Pc-G* and *trx* are the homeotic (*HOM-C*) genes in the *Antennapedia* and *bithorax* complexes. Drosphila *trx* mutants show loss or posteriorly shifted patterns of *HOM-C* gene expression that result in defects in segment identity, indicating *trx* is a positive regulator of *HOM-C* expression (6–8). In contrast, mutations in the *Pc-G* result in anterior shifts in *HOM-C* expression boundaries, indicating these proteins normally function as repressors of *HOM-C* expression (5). The data available suggest that *Pc-G* and *trx*-G proteins regulate gene expression by forming large multisubunit complexes at specific chromosomal sites. Subcellular localization studies show that *trx* and *Pc-G* proteins colocalize at many sites on polytene chromosomes, including the *bithorax* complex, the genes that are most affected by *Pc-G* and *trx* mutations (9). Further support for this concept comes from immunoprecipitation and fractionation studies that show that *Pc-G* members such as Polycomb and polyhomeotic are associated in multimeric protein complexes estimated to be in the 2–5 $\times$ 10^6 Da size range (10).

Many aspects of homeobox regulation appear to be conserved between *Drosophila* and mammals. In mammals, the clusterd homeobox (*Hox*) genes are organized in linear arrays on four different chromosomes. As in the fly, their 3′ to 5′ organization in the genome parallels their rostral to caudal anterior expression boundaries in the developing embryo. Recently, mammalian homologs of the *Pc-G* proteins have been identified, including *Bmi-1*, *Mph-1*, *EZH2*, and *M33* (11–15). Furthermore, some of these homologs have been shown to regulate *Hox* gene expression *in vivo*. Bmi-1 was first identified as a collaborator in c-*myc*-induced murine lymphomogenesis and is homologous to the Drosophila *Pc-G* member posterior sex combs (12, 13). Mice with homozygous disruption of *Bmi-1*, the mammalian homolog of *Posterior sex combs*, show anterior shifts in *Hox* expression boundaries (16, 17).

Mll-deficient mice demonstrate altered *Hox* expression and abnormal segmental identity. Of note, the *Mll* heterozygous mice show an overt phenotype. Skeletal abnormalities were frequently noted in heterozygous mice. $Mll^{+/-}$ newborns revealed frequent homeotic transformations, including posterior T12 $\rightarrow$ L1 transformations, as indicated by complete or partial loss of the T13 rib as well as anterior transformations (C7 $\rightarrow$ C6, T3 $\rightarrow$ T2). Homeotic defects were observed in $+/-$ animals whether the disrupted allele was of maternal or paternal origin, arguing they were the result of haploinsufficiency rather than parental imprinting (18). Gene dosage effects have been reported in *trx* mutants in *Drosophila* as well as in *Bmi-1* mutant mice (19).

$Mll^{-/-}$ embryos were embryonic lethal and showed severe developmental abnormalities, including abnormal ganglia, absence of the maxillary branch of the first branchial arch, small fore and hind limbs, and pooling of erythroid precursors in the coelomic cavity (20). Viable embryos could be recovered up to embryonic day 10.5 (E10.5). Histologic sections of the *Mll* null embryos showed extensive apoptotic cell death that was most marked in the first branchial arch, somites, and liver. These

Abbreviations: MLL, mixed lineage leukemia; RT, reverse transcription; E10.5, embryonic day 10.5; MEF, murine embryonic fibroblast.

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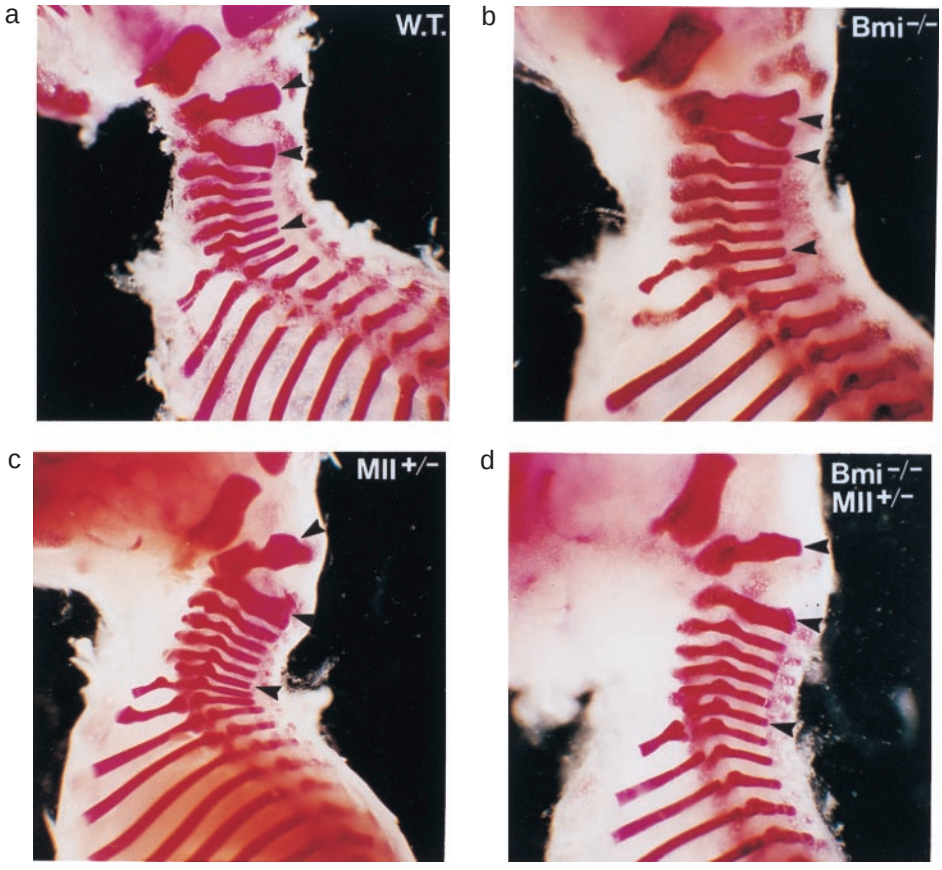

Fig. 1. Rescue of axial skeletal transformations in *Mll*, *Bmi-1* double-mutant mice. The cervical and anterior thoracic skeletons stained with alizarin red from E18 fetuses of the indicated genotypes are shown. Arrowheads mark the first, second, and seventh cervical vertebrae.

findings suggest that MLL or downstream target genes of MLL play a major role in regulating both cell proliferation and survival in the developing embryo. The *Mll* knockout indicated that MLL is required for the maintenance rather than the initiation of gene expression in early embryogenesis. Genes downstream of *Mll* including *Hoxa7* are activated appropriately in the absence of *Mll* but require *Mll* for sustaining their expression (20). Given that both *MLL* and *Bmi-1* are involved in hematopoietic malignancies, we chose to examine whether *Bmi-1* and *Mll* are antagonistic in the same developmental regulatory pathways.

Materials and Methods

$Mll^{+/-}$ and $Bmi\text{-}1^{+/-}$ mice have been described (18, 19). *Mll*-mutant mice used in this study have been backcrossed for more than 10 generations on a C57BL6/C3H backgound. *Bmi-1*-mutant mice have been maintained since their initial production on an FVB background. Mice heterozygous for both *Mll* and *Bmi-1* were mated to obtain animals for this study. Genotyping was done by using tail DNA (18, 19) as described. Skeletons were prepared as described (21).

The *Hoxc8* probe is a 408-bp reverse transcription (RT)–PCR product (nucleotides 274–682) cloned into the *Hinc*II site of pBS-SK. The plasmid was linearized with *Kpn*I and an antisense transcript synthesized with T3 RNA polymerase. *In situ* hybridization was as described (22).

Bmi-1 cDNA was cloned by RT-PCR using mouse fibroblast RNA and together with a 5′ HA tag was cloned into pUHD15–1 expression vector (www.zmbh.uni-hcidelberg.de/bujard/homepage.html). The *MLL* expression plasmid is described (23, 24) with the modification of a 5′ FLAG epitope.

Murine embryonic fibroblast (MEF) lines were established from E10.5 C57BL6/C3H embryos and were immortalized by transfection with a Polyoma virus expression plasmid (25). Cells were plated in 6-well plates and were transfected with 1 μg of total DNA by using Lipofectamine Plus (GIBCO/BRL). Stable transfectants were selected in media containing 135 μg/ml hygromycin. Twenty to thirty colonies were typically obtained from each transfection. RNA was isolated with RNeasy miniprep columns (Qiagen, Chatsworth, CA) and was treated for 15 min with DNase I (GIBCO/BRL, amplification grade). RNA (400 ng) was used for RT-PCR. Sequences of the oligonucleotide primers will be provided on request. 293 cells were transiently transfected by using Lipofectamine (GIBCO/BRL). Cells were permeabilized and stained with mouse M2 FLAG monoclonal antibody (Sigma) and rabbit anti-HA antibody (Santa Cruz Biotechnology) at 10 μg/ml. Secondary detection was with goat anti-mouse Alexa 488 (Molecular Probes) and donkey anti-mouse Cy3 conjugates (Jackson ImmunoResearch) at 1:250. Images were acquired on a Zeiss/Molecular Dynamics laser scanning confocal microscope, and fluorescence intensities were adjusted by using Molecular Dynamics IMAGE SPACE software.

Results

Mll heterozygous and *Bmi-1* heterozygous mice were mated to obtain double mutant mice, and the skeletal phenotype was characterized. Abnormalities in the cervical spine of $Bmi\text{-}1^{-/-}$ mice include a widened and split first cervical vertebra (C1), a small C2 suggestive of C2 → C3 transformation, and an ectopic rib associated with C7, indicative of C7 → T1 transformation (Fig. 1 *a* and *b*). $Mll^{+/-}$ cervical skeletons are notable for frequent widening of C2 (Fig. 1*c*). Compound double-mutant

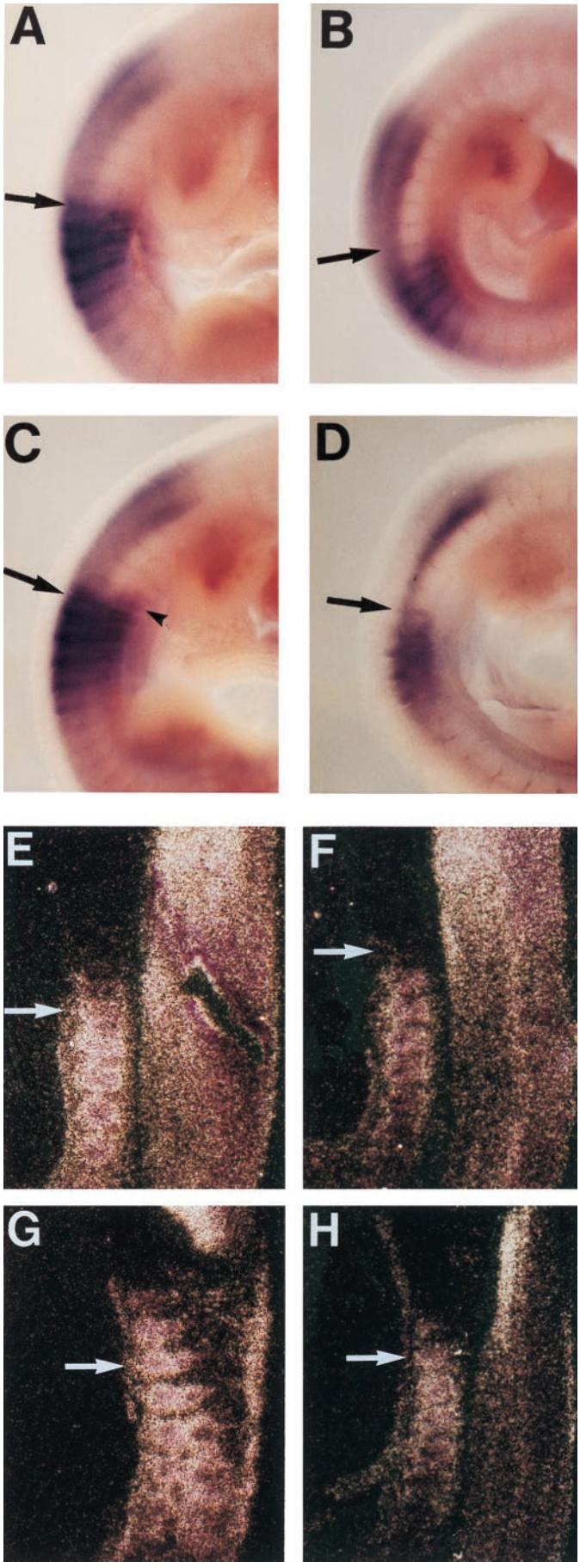

Table 1. *Bmi*/*Mll skeletal analysis*

	Mll$^{+/-}$, *historical**	*Mll*$^{+/-}$	Bmi$^{-/-}$, historical*	Bmi$^{-/-}$	Mll$^{+/-}$ Bmi$^{-/-}$
Widened/ split C1	N.R.	0/8	13/15	4/5	0/8
Widened/ split C2	14/22	6/8	3/15	0/5	2/8
C → C3	0/22	0/8	N.R.	4/5	0/8
C7 → T1	0/22	0/8	9/15	4/5	2/8

N.R., not reported.

*Mice used in this study were compared to historical controls (18, 19) to rule out strain-dependent modifiers of phenotype.

mice (*Bmi-1*$^{-/-}$, *Mll*$^{+/-}$) corrected several *Mll*- and *Bmi-1*-deficient phenotypes (Fig. 1*d*; Table 1), of particular note being the widened/split C1 and C2 → C3 transformation of *Bmi-1* deficiency. Thus, a balanced loss of these homeotic regulators results in a normalization of segment specification. The frequent abnormalities of the sternum, sternebrae, and thoracolumbar spine found in the individual gene mutants were not reduced in the double mutants (data not shown). This suggests that *Mll* and *Bmi-1* do not universally co-regulate the same homeotic genes at every segmental level.

We searched for target genes co-regulated by *Mll* and *Bmi-1* by assessing the expression patterns of the *Hox* clusters. Specifically, we analyzed *Mll*$^{+/-}$, *Bmi-1*$^{-/-}$ double mutant embryos to determine whether *Bmi-1* is the specific *Pc-G* member responsible for repressing the anterior limit of *Hox* expression in *Mll* mutants and conversely whether *Mll* is required for the ectopic *Hox* expression observed in *Bmi-1* mutants. Importantly, a one-segment posterior shift of *Hoxc8* observed in E9.5 and E12.5 *Mll*$^{+/-}$ embryos (Fig. 2 *B* and *F*) and a one-segment anterior shift in *Bmi-1*$^{-/-}$ embryos (Fig. 2 *C* and *G*) were normalized in the double mutants (Fig. 2 *D* and *H*). This result indicates that MLL and BMI-1 exert their effects in the segments immediately adjacent to the normal boundary of *Hoxc8* expression and can be unmasked when the opposing factor is reduced or absent. Of note, the intensity of staining for *Hoxc8* transcripts was reproducibly higher in *Bmi-1*$^{-/-}$ embryos and lower in *Mll*$^{+/-}$ and *Mll*$^{+/-}$, *Bmi-1*$^{-/-}$ embryos, suggesting a dominance of MLL in transcriptional control.

To further explore the effects of MLL versus BMI-1, we examined the expression of endogenous *Hox* genes in MEFs. *Mll*-deficient, as compared with wild-type MEFs, displayed either no or markedly decreased expression of the *Hox c* cluster (*c4*, *5*, *6*, *8*, *9*) and the *Hox a* cluster (*a3*, *4*, *5*, *7*, *9*, *10*) (Fig. 3*a*). In contrast, *Bmi-1*-deficient MEFs demonstrated elevated levels of *Hoxc6* and *c8* transcripts but no alteration of *Hoxa5*,*9*,*10* expression (Fig. 3*b*). This indicates that selected *Hox*, including *c8*, but not all *Hox* genes, are reciprocally regulated by MLL and BMI-1.

We next wished to reconstitute in a chromatin environment the differential regulation of a physiologic target gene, *Hoxc8*, by the *Pc-G* and *Trx-G* members *Mll* and *Bmi-1*. Studies of *Hoxc8*

Fig. 2. Rescue of *Hoxc8* deregulation in *Mll*/*Bmi-1* double-mutant embryos. (*A–D*) *Hoxc8* whole-mount *in situ* hybridization of E9.5 embryos. (*E–H*) *In situ* hybridization sections of E12.5 embryos. Arrows indicate the wild-type boundary of expression: specifically, the anterior edge of somite 14 (*A–D*) and the 12th prevertebral body (*E–H*). A posterior shift and decreased expression of *Hoxc8* were observed in Mll$^{+/-}$ embryos (*B* and *F*). An anterior shift of *Hoxc8* boundaries in somites and presomitic mesoderm (arrowhead in *C*) was seen in *Bmi-1*$^{-/-}$ embryos (*C* and *G*). The *Mll*$^{+/-}$*Bmi-1*$^{-/-}$ double mutants (*D* and *H*) restored the anterior boundaries to wild-type positions (*A* and *E*) but displayed decreased levels of expression.

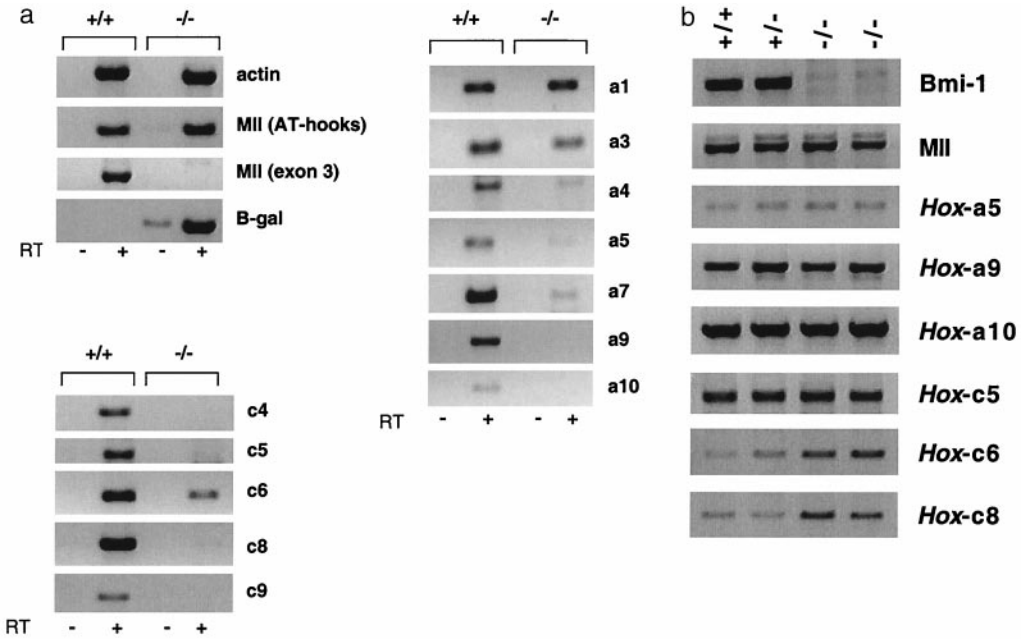

Fig. 3. Hox expression in MEFs. (*a*) MEF lines prepared from *Mll*$^{+/+}$ and *Mll*$^{-/-}$ embryos were examined for their expression pattern of *Hoxa* and *c* genes by a semiquantitative RNA RT-PCR analysis. *Mll* (AT-hooks) region is present in the disrupted (−/−) as well as wild-type (+/+) alleles whereas *Mll* (exon 3) is deleted, but β-galactosidase is inserted in the (−/−) alleles. RT signifies the presence (+) or absence (−) of reverse transcriptase. (*b*) *Hoxa* and *c* expression in *Bmi-1*$^{+/+}$ versus *Bmi-1*$^{-/-}$ MEFs.

expression during embryogenesis have identified critical regulatory regions upstream and downstream of the gene (26, 27). The 5′ element is necessary and sufficient for correct temporal and tissue-specific activation of *Hoxc8* expression but by itself is unable to direct expression to the correct anterior boundaries beyond E9 and fails to maintain expression beyond E12. The 3′ regulatory element is required for appropriate anterior boundaries and maintains expression throughout embryogenesis. Consequently, we used a reporter construct in which lacZ expression is directed by the *Hoxc8* promoter, 5′ and 3′ enhancer elements (Fig. 4*a*). As might be predicted, *Mll* status did not affect the expression of an extrachromosomal construct in transient expression assays (data not shown). In contrast, genomic integration of the *Hoxc8*-lacZ gene in stable transfectants provided a

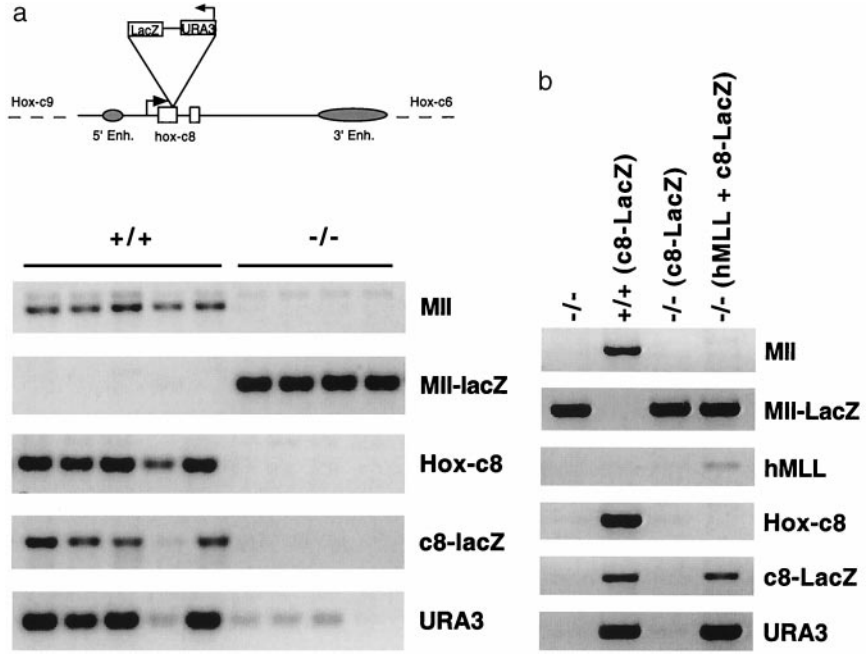

Fig. 4. Expression of *Hox c8-lacZ* is reserved by *MLL*. (*a*) MEFs from *Mll*$^{+/+}$ and Mll$^{-/-}$ embryos were transfected with a *Hoxc8-lacZ* construct, demonstrating integration site independent regulation of Hoxc8 in stable transfectant clones. (*b*) Expression of human *MLL* (*hMLL*) results in the expression of the integrated *Hoxc8-lacZ* reporter but not the endogenous *Hoxc8* locus.

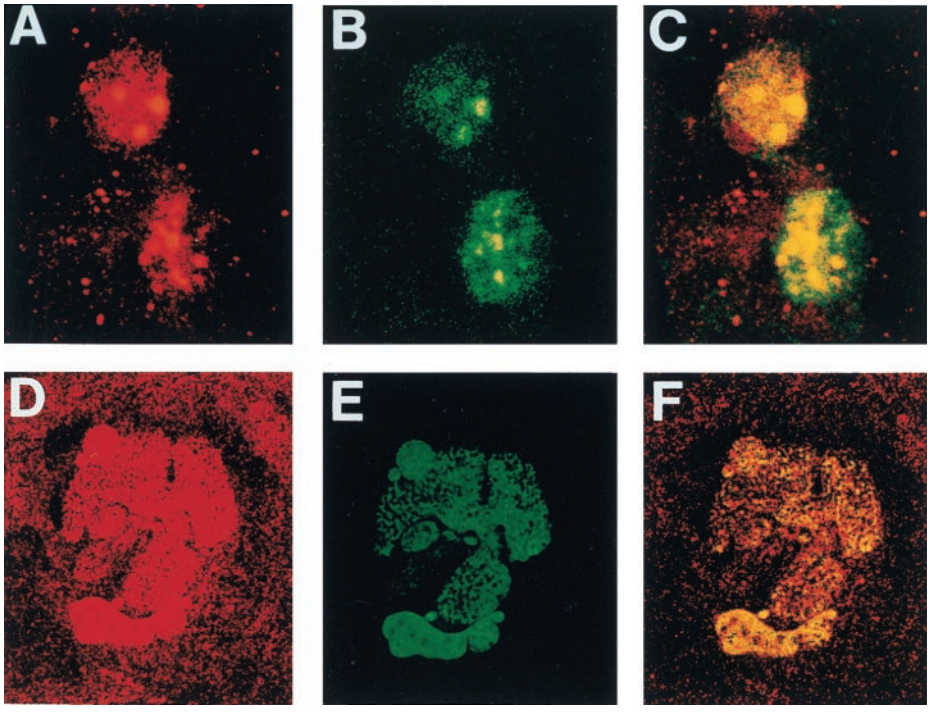

Fig. 5. Bmi-1 and MLL expression plasmids produce proteins with discrete subnuclear colocalization. (*A–C*) Transiently transfected 293 cells. (*D–F*) MEF stable transfectants were stained for HA-tagged BMI-1 (*A*, *C*, *D*, and *F*) and FLAG-tagged MLL (*B*, *C*, *E*, and *F*). Laser confocal microscopy demonstrates discrete subnuclear localization of both proteins with significant colocalization when the images are superimposed (*C* and *F*, yellow fluorescence).

chromatin context that proved permissive for MLL regulation. The *Hoxc-8*-lacZ reporter, like the endogenous *Hoxc8* locus, was repressed in *Mll*-deficient MEFs (Fig. 4*a*). However, four of five $Mll^{+/+}$ clones expressed the stably integrated *Hoxc8*-lacZ reporter (Fig. 4*a*).

One model holds that Pc-G activity increases the probability of forming a repressed chromatin conformation. Consequently, we asked whether *MLL* overexpression would antagonize the tendency for repression by an unopposed *Pc-G*, presumably *Bmi-1*, in $Mll^{-/-}$ cells. Cells transfected with an *MLL* expression vector were found to now express *Hoxc8-lacZ* whereas the endogenous *Hoxc8* locus could not be activated (Fig. 4*b*).

To assess whether MLL and BMI-1 show any evidence for colocalization that would enable them to regulate shared loci, laser confocal immunomicroscopy was used. Transient cotransfection of *MLL* and *Bmi-1* into mammalian 293 cells demonstrated a nuclear localization for both proteins that consists of numerous discrete speckles, as well as 5–10 larger aggregates per cell (Fig. 5 *A* and *B*). Exact alignment of doubly stained larger aggregates was consistently observed whereas colocalization was also noted for the majority of the smaller speckles (Fig. 5*C*). We also verified localization in stable transfectants, where MLL and BMI-1 expression is similar to endogenous levels. In this case, the vast majority of MLL and BMI-1 was present as discrete speckles (Fig. 5 *D* and *E*), over half of which displayed colocalization (Fig. 5*F*).

Discussion

Initiation of *Hox* expression in *Drosophila* is under the control of the segmentation genes, which function to activate or repress specific *Hox* gene transcription in the appropriate segments (5). These factors are short-lived, and maintenance of the established patterns is controlled by the *Trx* and *Pc-G* members. Double mutants of Trx-G and Pc-G in Drosophila were noted to restore a wild-type phenotype (28). Substantial data supports the view that these ubiquitously expressed homeotic regulators act differentially on specific promoters in specific cell types to maintain the initially established transcriptional state (2–5). Axial *Hox* expression patterns in early mouse development argues that MLL may be critical for maintenance rather than initiation of *Hox* transcription (20). However, *Hox* transcription continues to be modulated throughout mammalian development. Hematopoietic cells in particular demonstrate ongoing activation and repression of *Hox* expression at each maturation point and in all hematopoietic lineages (29). The relative expression of Pc-G transcripts in early hematopoietic precursors has been shown to change during maturation with both up- and down-regulation of individual *Pc-G* genes (30).

The axial–skeletal transformations and altered *Hox* expression patterns of *Mll*-deficient and *Bmi-1*-deficient mice were normalized when both *Mll* and *Bmi-1* were deleted, demonstrating their antagonistic role in determining segmental identity. Moreover, we noted that selected *Hox*, including *c8*, but not all *Hox* genes, are reciprocally regulated by MLL and BMI-1. An experimental model is needed to further refine cis-acting elements and pursue their differential regulation by an MLL or *Bmi-1* associated multimeric protein complex. We sought a target gene regulated reciprocally by the *Trx-G* member *Mll* and selected *Pc-G* member *Bmi-1*, as they are both involved in oncogenesis. *Mll*-deficient and *Bmi-1*-deficient MEFs provide an easily obtainable and replenishable source of null cells that were assessed for their expression of the clustered *Hox* genes. *Hox-c8* proves an attractive candidate to further define critical regulatory sequences as it was clearly reciprocally affected by the status of *Mll* and *Bmi-1*.

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